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Structural Requirements of Double-stranded RNA for the Activation of 2′,5′-Oligo(A) Polymerase and Protein Kinase of Interferon-treated HeLa Cells*

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Addition of double-stranded RNA (dsRNA) to extracts of interferon-treated HeLa cells results in the synthesis of 2′,5′-oligo(A) from ATP and in the phosphorylation of a ribosome-associated protein of M_r = 72,000. Previously described assays were used to investigate the structural requirements of dsRNA for the activation of these two enzymatic activities. Poly(CG) with different ratios of C/G was synthesized with polynucleotide phosphorylase. These polynucleotides were either annealed with poly(I) to form mismatched dsRNA or digested with ribonuclease T_1 to produce smaller polynucleotides. Polymers with an average of one mismatch every eight nucleotides failed to activate the 2′,5′-oligo(A) polymerase and protein kinase, whereas polymers with a mismatch every 45 nucleotides were fully active. The polynucleotides obtained by T_1 digestion of poly(CG) were fractionated by gel filtration into discrete size polymers. These sized polynucleotides were annealed with high molecular weight poly(I) and assayed for activation of 2′,5′-oligo(A) polymerase and protein kinase. These enzymes could not be activated by dsRNA containing poly(C) shorter than 30 nucleotides. Maximal activation was obtained with dsRNA containing poly(C) longer than 65 to 80 nucleotides. A similar size requirement for activation was observed with dsRNA formed with poly(A) and poly(U) of known length. These results indicate that a relatively long stretch of base pairs, uninterrupted by either a mismatch or a discontinuity in one of the complementary strands, is required for the activation of the two enzymes studied. These structural characteristics are similar to those previously shown to be required for the induction of interferon by dsRNA.

Natural and synthetic dsRNAs[1] are among the most potent interferon inducers (1). Furthermore, dsRNA is also a potent inhibitor of protein synthesis in extracts of interferon-treated cells (2). This inhibition is due to elevated levels of a protein kinase and an oligonucleotide polymerase which require dsRNA, ATP, and Mg^{2+} for activity (see Ref. 3). Activation of the protein kinase by dsRNA results in the phosphorylation of a polypeptide of about M_r = 70,000 and of the α subunit of initiation factor eIF-2 (3, 4). The oligonucleotide polymerase, designated 2′,5′-oligo(A) polymerase (3), synthesizes from ATP a series of oligonucleotides containing the unusual 2′,5′-phosphodiester linkage (5). The 2′,5′-oligo(A) is not itself inhibitory to protein synthesis, but activates an endonuclease present in both control and interferon-treated cells and inhibition of protein synthesis occurs via mRNA degradation (6, 7).

Relatively little is known about the molecular features of dsRNA which are required for the activation of the protein kinase and 2′,5′-oligo(A) polymerase. RNA/DNA hybrids and the triple-stranded polymer poly(A)·poly(U)·poly(U) do not activate the latter enzyme (8). In contrast, there is vast literature on the molecular features of dsRNA which are relevant for the induction of interferon (1). For example, the presence of mismatched nucleotides in synthetic dsRNA causes a loss of activity (9) and the dsRNA must be longer than approximately 50 base pairs to induce interferon (10).

We report here the results of an investigation of the effect of mismatched nucleotides and of polymer size on the activation of 2′,5′-oligo(A) polymerase and protein kinase by dsRNA. Interestingly, the structural features of dsRNA relevant for the activation of these enzymes are similar to those reported in the literature for the induction of interferon.

EXPERIMENTAL PROCEDURES

Chemicals—Radiochemicals were purchased from New England Nuclear; sized poly(A) and poly(U) from Miles; polynucleotide phosphorylase from P-L Biochemicals; nuclease T_1 from Sigma.

Cells, Extracts, and Assays—HeLa cells grown in suspension culture were treated with 100 reference units/ml of human fibroblast interferon (3×10^5 units/mg) for 17 h prior to harvest. Interferon was obtained from the Interferon Working Group, National Cancer Institute, NIH. Extracts were prepared from these cells as previously described (8). Ribosomes were isolated by centrifugation and resuspended as described (11). Protein kinase activity was determined in assays containing 0.1 mM [γ-^{32}P]ATP (1.3 Ci/mol) and 30 to 40 μg of ribosomes in a final volume of 30 μl. Incubation was at 30°C for 7 min prior to fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (11). Autoradiographs of the gels were scanned at 560 nm, and the area under the peak corresponding to a polypeptide of M_r = 72,000 was measured as previously described (12). Assays for the synthesis of 2′,5′-oligo(A) contained unless otherwise indicated 5 μl of cell extract (about 10 mg protein/ml), 0.12 M KOAc, 25 mM $Mg(OAc)_2$, 20 mM Hepes/KOH, pH 7.4, 5 mM [^{3}H]ATP (1.6 Ci/mol), 4 mM fructose 1,6-bis-phosphate, 1 mM dithiothreitol, and the indicated amount of dsRNA in a final incubation volume of 25 μl. Incubation was at 30°C for 60 min and reactions were terminated by heating to 95°C for 3 min. The 2′,5′-oligo(A) formed was determined by chromatography on DEAE-cellulose as previously described (8).

Preparation of CG Copolymers, Sized Polynucleotides, and Double-stranded RNA—Ribonucleotide diphosphates were polymerized with *Micrococcus lysodeikticus* polynucleotide phosphorylase (2 mg/ml) at 37°C for 2 h as described (9). Reactions contained CDP and [^{3}H]GDP in molar ratios of 10:1 to 200:1. Polynucleotides were extracted with phenol and dialyzed exhaustively (9). The C and G

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[1] The abbreviations used are: dsRNA, double-stranded RNA; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

content of polynucleotides was calculated from the A_{271}/counts per min ratio of dialyzed material digested overnight with 0.3 N KOH by comparison of this ratio with that of the unpolymerized starting material. Sized polynucleotides were prepared from poly(CG) by 3-h digestion at 37°C with 0.5 to 1 unit of ribonuclease T_1 per A_{271} unit. The digestion products were applied to columns of Sephadex G-200, G-150, or G-50, depending on the expected size of the fragments generated. The size of the polynucleotides in the eluted fractions was estimated from the A_{268}/counts per min ratio. Correction was made for variation of extinction coefficient with chain length by assuming a negligible contribution to absorbance by the G residue present in the polynucleotides and taking absorbance values for oligo(C) from Adler *et al.* (13). Double-stranded RNA was formed by heating equimolar nucleotide amounts of complementary RNA species to 70°C in a buffer containing 0.1 M KOAc and 20 mM Hepes/KOH, pH 7.4, and cooling to 30°C. Poly(I) of M_r > 100,000 ($s_{20,w}$ = 9.4) was used.

RESULTS

Effect of Base Mismatching on the Activation of 2',5'-Oligo(A) Polymerase and Protein Kinase—Polyribonucleotides containing different ratios of C to G were synthesized with polynucleotide phosphorylase. These polynucleotides were annealed with poly(I) to form dsRNA containing mismatched bases, since G cannot form a normal Watson-Crick base pair with I. The relative mismatching of these dsRNAs is inversely proportional to the C/G ratio. The dsRNAs are designated by the C/G ratio of the poly(CG) strand.

Polymers with a C/G ratio of 7 do not promote synthesis of 2',5'-oligo(A) or phosphorylation of the M_r = 72,000 polypeptide (Figs. 1; 2, *A* and *D*). Polymers with a C/G ratio of 15 are partially active in both assays and polymers with a ratio of 45 or higher are fully active.

The effect of increasing concentrations of polymer on the synthesis of 2',5'-oligo(A) was next investigated. Polymers with a C/G ratio of 45 show maximum activity at 5 μg/ml, whereas a polymer with a ratio of 7 shows less than 5% of this activity at 20 μg/ml (data not shown). The activity of polymers of different C/G ratio cannot be explained by differential degradation of these polynucleotides. No degradation to acid-soluble material was detected when polymers of C/G ratio of 7 and 15 were incubated for 90 min under the conditions of our assays (data not shown). The simplest interpretation of the above results is that a minimum length of perfectly matched I·C base pairs is necessary for activation of 2',5'-oligo(A) polymerase and protein kinase.

Size Requirements for Activation of 2',5'-Oligo(A) Polymerase and Protein Kinase—Polynucleotides of different C/G ratios were digested with ribonuclease T_1 to yield a series of poly(C) fragments terminating in G (9). These fragments were fractionated by gel filtration and their size determined as described under "Experimental Procedures." In order to generate a full series of sized fragments, polynucleotides with C/G ratios of 200, 45, and 20 were digested and fractionated in this way. Sized poly(C) fragments ranging in average length between 10 and 380 nucleotides were thus obtained. These sized polynucleotides were annealed with equimolar nucleotide amounts of poly(I). The dsRNAs formed are designated as $I_n \cdot C_x$, where x indicates the average number of C residues of a sized polynucleotide terminating in G. The dsRNAs were assayed for activation of 2',5'-oligo(A) polymerase and protein kinase (Figs. 1 and 2, *B* and *E*). Both enzymes were activated only by dsRNAs containing poly(C) longer than 35 nucleotides, with maximal activity being observed with $I_n \cdot C_{65}$ for synthesis of 2',5'-oligo(A) and with $I_n \cdot C_{80}$ in the kinase assay.

Partial activation of 2',5'-oligo(A) polymerase and protein kinase by dsRNAs containing poly(C) 35 to 50 nucleotides long could be due to contamination of the sized polynucleotides with poly(C) of greater length. To test this possibility, we determined the effect of increasing concentrations of dsRNA on the activation of 2',5'-oligo(A) polymerase (Fig. 3*A*). The polymer $I_n \cdot C_{32}$ was inactive below 10 μg/ml, but at 20 μg/ml had 3% of the activity of $I_n \cdot C_{55}$. We cannot distinguish, however, between a 3% contamination of C_{32} with longer poly(C) and a marginal activation of the enzyme by $I_n \cdot C_{32}$.

In the experiments described above, the 2',5'-oligo(A) polymerase assay was carried out at 20 mM $Mg(OAc)_2$ which gives optimal synthesis of 2',5'-oligo(A) with HeLa cell extracts (8). When 2',5'-oligo(A) synthesis was assayed at the same $Mg(OAc)_2$ used in the kinase assay (2 mM), an increase in the size of dsRNA required for activation was observed. The polymer $I_n \cdot C_{37}$ showed little activity and $I_n \cdot C_{32}$ was

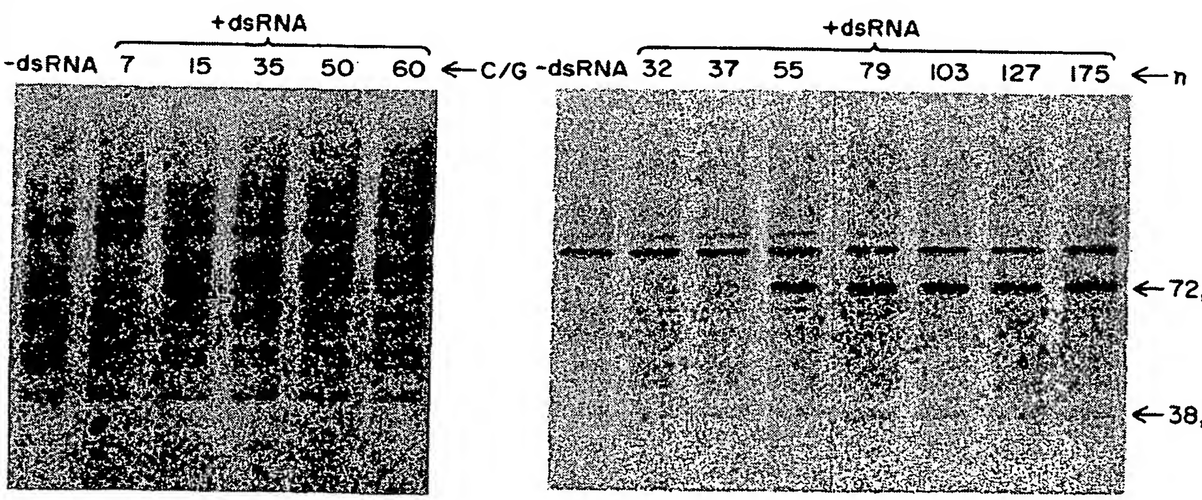

FIG. 1. Phosphorylation of the M_r = 72,000 polypeptide by the protein kinase activated with poly(I)·poly(CG) (*left*) or by poly(I) annealed to sized poly(C) (*right*). Ribosomes from interferon-treated HeLa cells were incubated with or without 0.5 (*left*) or 0.1 (*right*) μg/ml of dsRNA for 7 min at 30°C and analyzed by gel electrophoresis and autoradiography as described under "Experimental Procedures." The tracks show from *left* to *right*: an incubation without added dsRNA; incubations with added poly(I)·poly(CG), with the ratio C/G of the polymer indicated for each track; an incubation without added dsRNA; incubations with added poly(I) annealed to poly(C) of the length indicated for each track (*n*). The position of the M_r = 72,000 and 38,000 polypeptides is indicated on the *right*.

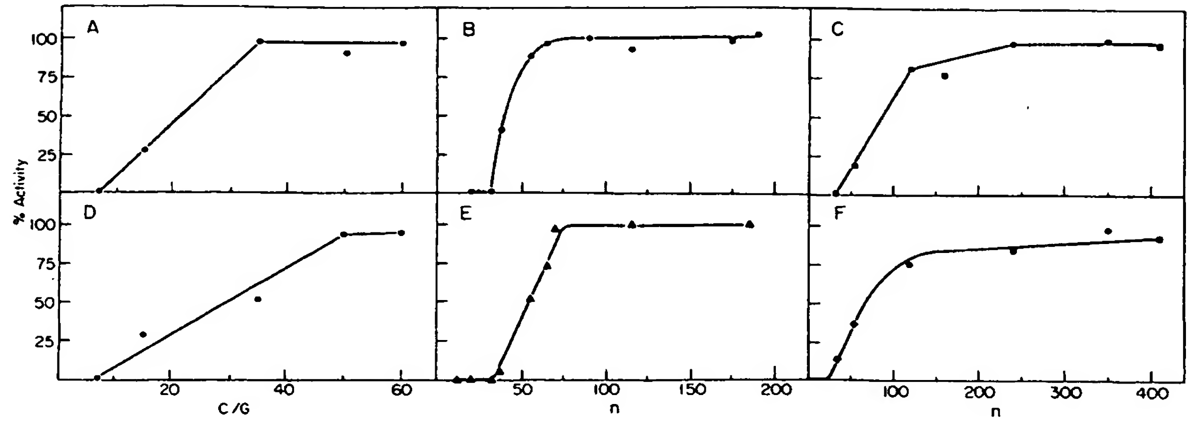

FIG. 2. **Synthesis of 2',5'-oligo(A) (*A*, *B*, and *C*) and phosphorylation of the M_r = 72,000 polypeptide (*D*, *E*, and *F*) promoted by poly(I)·poly(CG) with different ratios of C/G (*A* and *D*), by poly(I) annealed to sized poly(C) (*B* and *E*), and by sized poly(A) annealed to sized poly(U) (*C* and *F*).** Preparation of polymers and assays for 2',5'-oligo(A) polymerase and protein kinase are described under "Experimental Procedures." The polymers were tested at 10 μg/ml in the polymerase assay and at 0.5 (*D*) or 0.1 (*E* and *F*) μg/ml in the kinase assay. The activity is expressed as a percentage of that obtained with poly(I)·poly(C). With this polymer, 33 nmol of ATP were converted to 2',5'-oligo(A) from an input of 125 nmol of ATP per reaction. In the kinase assay, phosphorylation was determined by scanning autoradiographs of gels, like those shown in Fig. 1, and measuring the area under the M_r = 72,000 band. Phosphorylation observed with different polymers is shown as a percentage of that obtained with poly(I)·poly(C). On the *abscissa* is indicated the C/G ratio of the polymers tested (*left panels*), the length in nucleotides of the poly(C) annealed to poly(I) (*middle panels*), and the length of the shorter polynucleotide used to form dsRNA with sized poly(A) and poly(U) (*right panels*). Different symbols designate in these latter panels the longer complementary polynucleotides annealed: (●) A_{410}, (◆) U_{120}, and (■) U_{500}.

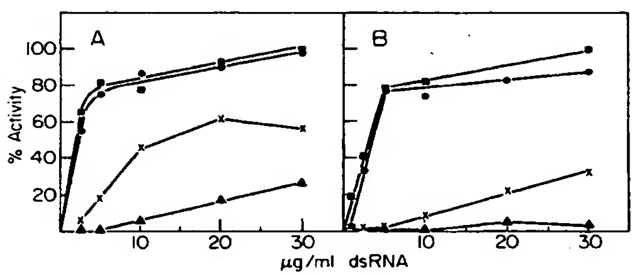

FIG. 3. **Activation of 2',5'-oligo(A) polymerase by different concentrations of poly(I) annealed to sized poly(C) with (*A*) 20 mM and (*B*) 2 mM Mg(OAc)₂.** Increasing concentration of poly(I)·C_{32} (▲——▲), poly(I)·C_{37} (×——×), poly(I)·C_{55} (●——●), and poly(I)·C_{380} (■——■) were added to standard incubation mixtures. With 30 μg/ml of poly(I)·poly(C) and 20 mM Mg(OAc)₂, 40 nmol of ATP were converted to 2',5'-oligo(A) from an input of 125 nmol, whereas 20 nmol were converted with 2 mM Mg(OAc)₂. Each reaction contained also 5 mM Mg(OAc)₂ added in equimolar amount with ATP (see "Experimental Procedures"). The synthesis of 2',5'-oligo(A) is expressed as a percentage of that obtained with 30 μg/ml of poly(I)·poly(C).

practically inactive even at 30 μg/ml (Fig. 3*B*), whereas I_n·C_{55} remained fully active.

The failure of dsRNAs containing short poly(C) to activate either the 2',5'-oligo(A) polymerase or the protein kinase may be due to low affinity of the enzymes for these polymers. Alternatively, these dsRNAs may bind the enzymes but may not function as activators. This dsRNA/enzyme interaction was studied by competition experiments, in which an active dsRNA was assayed in the presence of increasing amounts of inactive dsRNA. No competition between 2.5 μg/ml of active dsRNA and 30 μg/ml of inactive dsRNA was detected in the 2',5'-oligo(A) polymerase assays (data not shown). The results of experiments testing competition in the protein kinase assay were more difficult to interpret. Activation of the kinase occurs only within a range of dsRNA concentrations, and high dsRNA levels are inhibitory (14). Addition of a 10-fold excess of I_n·C_{30} had no effect on kinase activation by 0.1 μg/ml of I_n·C_{130}, whereas a 100-fold excess significantly impaired kinase activation. The concentration of dsRNA was raised in this case to 10 μg/ml. Both short and long polymers were inactive at this dsRNA concentration (Ref. 11 and data not shown). It seems possible, therefore, that inactive dsRNAs may prevent activation of the kinase by raising the dsRNA concentration to the inhibitory range.

Sized poly(A) and poly(U) were similarly tested upon annealing with equimolar nucleotide amounts of complementary strands. It was therefore possible to construct dsRNAs with two polynucleotides of known length. The dsRNAs containing A_{33} were inactive in the 2',5'-oligo(A) polymerase assay and only slightly active in the kinase assay (Fig. 2, *C* and *F*). The dsRNAs containing A_{54} were partially active in both assays, and those containing U_{120} annealed to longer poly(A) were fully active. The effect of the complementary chain length on the activation of 2',5'-oligo(A) polymerase was investigated in a systematic way (Table I). The activity of the dsRNAs was found to be dependent mainly on the length of the shorter polynucleotide. The length of the complementary polynucleotide is also important, however, as shown by the higher activity of A_{54} annealed with poly(U) of increasing length. A

TABLE I

Effect of varying poly(A) and poly(U) size on the synthesis of 2',5'-oligo(A)

Polynucleotide[a] (average size)		2',5'-Oligo(A) synthesized
A	U	*nmol adenosine polymerized*
33	120	0
54	120	0.7
54	240	1.4
54	500	6.3
160	120	14.3
160	350	19.1
160	500	25.5
410	240	32.5
410	500	31.7

[a] Equimolar nucleotide amounts of poly(A) and poly(U) were annealed and assayed at 20 μg/ml as described under "Experimental Procedures." Each reaction contained 125 nmol of [³H]ATP.

possible explanation for this effect is that annealing of two relatively short polymers is likely to result in more discontinuities in complementary strands than when a shorter polymer is annealed with a longer one. More double-stranded sequences of the minimal length necessary for enzyme activation will be formed in this latter case.

DISCUSSION

We have studied the activation of two dsRNA-dependent enzymatic activities by preparations of poly(I)·poly(CG) containing different proportions of G residues (mismatched dsRNA). The results obtained clearly indicate that a minimum length of perfectly matched base pairs is necessary for the activation of these enzymes. This length can be roughly estimated by calculating the average frequency of C runs of different length in polymers with variable degree of mismatching. If a random distribution of C and G residues is assumed to occur in these polymers, the probability of finding a C run of length n is then given by $(C/(C+G))^n$ (where $(C/(C+G))$ is the relative proportion of C in the mismatched strand). Runs of 35 or more C's are 40-fold more frequent in a polymer with a C/G ratio of 35 than in a polymer with a ratio of 7. Experimentally, the former polymer was found to be at least 20-fold more active than the latter polymer in promoting synthesis of 2′,5′-oligo(A).

A direct estimate of the dsRNA size requirement for activation of these enzymes was obtained by forming polymers with a high molecular weight poly(I) strand annealed to poly(C) of different length. Only dsRNA containing poly(C) longer than 65 to 80 nucleotides was fully active. These results were confirmed with poly(A) and poly(U) of known size annealed in different combinations. The structural requirements of dsRNA for activation of the 2′,5′-oligo(A) polymerase and protein kinase are therefore similar. There are, however, some differences between these two enzymes. The 2′,5′-oligo(A) polymerase is fully activated by poly(I)·poly(CG) with fewer mismatches than the kinase, and dsRNA of greater size is required for activation of this latter enzyme. These differences may in part be explained by the different Mg^{2+} concentration in the assays, since slightly larger dsRNA was required for maximal activation of 2′,5′-oligo(A) polymerase at lower Mg^{2+} concentration. The activation of these two enzymes differs in another way: the kinase cannot be activated in the presence of high concentrations of dsRNA (14), whereas the 2′,5′-oligo(A) polymerase is activated more effectively by high concentrations of dsRNA (8).

There are similarities between the structural requirements of dsRNA for interferon induction and those for activation of 2′,5′-oligo(A) polymerase and protein kinase. These similarities have been previously noticed in studies assaying the inhibition of protein synthesis by dsRNA in reticulocyte lysates (15) and extracts of interferon-treated L cells (16), presumably due to the combined action of the protein kinase and 2′,5′-oligo(A) polymerase/endonuclease system. A threshold molecular size of dsRNA corresponding to approximately 50 base pairs determines the interferon inducing activity of dsRNA (10). A similar size requirement is observed for the inhibition of protein synthesis in reticulocyte lysates by dsRNA (14). These observations agree with our findings that about 40 to 60 base pairs of dsRNA are required for the activation of 2′,5′-oligo(A) polymerase and protein kinase under different assay conditions. Similarly, the ability of dsRNA to induce interferon decreases with increasing content of mismatched bases (9, 17).

Our results are directly comparable to those obtained by Carter *et al.* (9) with poly(I)·poly(C_{20}G). This polymer can partially activate the 2′,5′-oligo(A) polymerase and protein kinase and has an intermediate interferon-inducing activity. Further work on the interferon-inducing activity of the other polymers used in our studies could possibly provide additional evidence for this correlation.

Some differences between the interferon-inducing activity and the inhibitory effect on protein synthesis of natural and synthetic dsRNA have been previously described (15, 16). Certain synthetic dsRNAs containing modified nucleotides are extremely efficient interferon inducers but do not significantly inhibit protein synthesis in reticulocyte lysates (15) or in extracts of interferon-treated L cells (16). Assays of these dsRNAs for activation of the 2′,5′-oligo(A) polymerase and protein kinase will establish how closely the interferon-inducing activity of dsRNA is correlated with its ability to activate these enzymes. It is tempting to speculate that a dsRNA-dependent interferon-induced enzyme is part of the cellular recognition system for dsRNA. Both 2′,5′-oligo(A) polymerase and protein kinase are present at a basal level in all mammalian and avian cells studied thus far (3). A dsRNA formed by a replicating virus or administered to cells may interact with these enzymes, which are known to bind dsRNA (18), and activate synthesis of 2′,5′-oligo(A). This compound may in turn have some biological role in the induction of interferon synthesis. Further studies are necessary to provide some experimental support for this working hypothesis.

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